THE PRESENCE OF GLYCOGEN IN THE CELLS OF EMBRYOS OF FUNDULUS HETEROCLITUS STUDIED IN TISSUE CULTURES.

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At the present time it is practically impossible to demonstrate the various chemical substances of the living cell, owing to the fact that most means of analysis cause the death of the cell. A few of the differential stains, such as Sudan III. and Nile blue, have been applied to studies of living tissues, but these do not behave in the same manner in the living cell as in dead material (Lewis and Lewis, 1915; M. R. Lewis, 1918). In most cases the chemical nature of cytoplasm has been discussed from the standpoint of results obtained from dead cells, and it is doubtful whether such conclusions can be applied directly to the living. This is true in regard to the experiments given herein; for, while the substance described reacts as does glycogen to the tests usually employed to demonstrate glycogen, these results were not obtained until after the cells had begun to be affected by the iodine.

Bernard (1859) demonstrated the presence of glycogen in many kinds of tissues and since that time several methods for showing this substance in the cell have been reported. The methods most frequently used for histological purposes are those of Best (1909), Gage (1917). Neither of these was used in the experiments given below. Instead, the living cells were exposed to iodine vapor in such a manner that they could be followed throughout the experiment, *i.e.*, when living, while dying, and after death had occurred.

Technique.

Small pieces of fundulus embryos (just before hatching) were explanted into hanging drops of the following solution: 80 c.c.

diluted (40%) sea water plus 0.02 per cent. $NaHCO_3$ plus 20 c.c. fundulus bouillon plus 0.5 per cent. dextrose (M. R. Lewis, 1917). Within 24 to 48 hours large growths were present around these explants. The cells composing the growths were then exposed to iodine vapor by scattering a few fragments of an iodine crystal in the bottom of the hollow ground slide under the hanging drop, care being taken to prevent them from touching the drop. The vapor from the iodine crystal penetrated the hanging drop and acted upon the cells. Just a sufficient amount of iodine should be used to rapidly color the cytoplasm yellow and to show the port-wine color of the glycogen within one to two minutes.

Normal Fundulus Cultures.

As has been shown by Dederer (1921), the ectoderm and the mesenchyme cells grow out from explants of fundulus embryos in the form of membranes. The mesenchyme cells are usually attached to the cover-slip and the ectoderm cells form a layer directly beneath them. Mesenchyme cells extend beyond the ectoderm and also along the edge of the membrane and those scattered farther out on the cover-slip are the only cells which have processes to any extent. These processes usually spread out at one end into a large, thin, fan-like structure. One or more large, flat, oval cells, probably endoderm, are sometimes found on the membranes; these are quite different from the mesenchyme cells, in that they have an oval shape while the mesenchyme cells are elongated or somewhat hexagonal, and their cytoplasm also appears to be of a different consistency. For convenience of description these cells will be called *oval* cells. When the cultures were placed over fragments of an iodine crystal the cytoplasm and nucleus of all the cells became yellow, the the mitochondria a darker yellow, and the fat globules a brownish color. Almost the entire cytoplasm of the oval cells, regions of the cytoplasm of some of the mesenchyme cells, and certain parts of a number of their processes turned a port-wine color. This was a distinctly different tint from that exhibited by any other part of the growth and was the same as the characteristic color exhibited by glycogen when exposed to the action of iodine.

The three regions which showed this color reaction will be discussed separately.

1. *Oval Cells.*—As the iodine vapor penetrated the hanging drop, the cytoplasm of these cells became a pale yellow; then the granular portion, including the mitochondria and fat droplets, *i.e.*, the endoplasm, appeared to shrink slightly and became a deeper yellow. Meanwhile the remainder of the cytoplasm, except a thin yellow ectosarc, turned pink, the color gradually deepening until, after a few seconds, this portion was a deep port-wine color, while the endoplasm, nucleus and ectosarc remained yellow. At times the port-wine-colored material occupied the greater part of the cell, leaving only a small clump of endoplasm and the nucleus. In other cases the yellow endoplasm, nucleus and ectoplasm took up practically the entire cell except what appeared as a large port-wine-colored vacuole. The contour of the cell did not change but remained the same size as it was before exposure to the iodine. In a few of these cells there were a number of vacuoles, which also became port-wine color. The color was more intense in tone in the oval cells than in any other portion of the growth. It remained in them for some time, in a few instances for over an hour, then faded, and the whole preparation became a dark yellow color.

2. *Mesenchyme Cells of the Membrane.*—Of the cells forming the membrane probably only the mesenchyme exhibited the port-wine color upon exposure to iodine. This was difficult to determine definitely because the cells adhered together so closely, and also because of the fact that, while the mesenchyme cells were frequently found extended beyond the ectoderm cells, the latter were never observed separated from the former. Not all of the mesenchyme cells showed the port-wine color when in the presence of iodine, and which ones would do so could not be foretold. In these cells the cytoplasm of the central and thicker portion of the cell became a diffuse pink color, while the nucleus and ectoplasm became yellow. This area of pink coloration was not definitely limited but toned off into an extensive yellow ectoplasm. The arrangement of the mitochondria and other structures was the same in the cells having a pink area as in

normal cells. Gradually the pink color became deeper in tone until it attained a pale port-wine color. Within a few seconds the color disappeared from the central region of the cell and a large, round port-wine-colored bleb appeared at one side of the cell. Practically no change was observed in the structure of the cell during the disappearance of the stain and the formation of the bleb. Many of these blebs appeared scattered over the membrane, especially in the region adjoining the explanted piece. The color remained in them for about twenty minutes and then faded out, leaving the blebs rather undefined and difficult to distinguish. In some of the more peripheral cells of the membrane the port-wine color remained diffuse in the cytoplasm of the central portion for some time and then faded out without forming blebs. The mesenchyme cells which had migrated out on the cover-slip, away from the membrane, seldom formed blebs.

3. *Cell Processes.*—The processes of the cells formed the region where the appearance of the port-wine color could be observed most clearly. The large fan-like processes referred to above exhibited lighter and darker areas where the cytoplasm varied either in density or in thickness. After exposure to iodine some of the lighter regions became pink. Later these turned into distinct port-wine-colored areas in the yellow cytoplasm. A few of the processes did not exhibit these stained areas at all; in others some of the areas remained quite pale in color. This phenomenon did not continue for longer than half an hour; at the end of this time the color had faded and that area of the process was slightly shrunken.

When a drop of saliva was placed upon a culture which was later exposed to iodine vapor the port-wine color was not found in the cells. Death of the cell also prevented its appearance. No granules having the characteristic glycogen color were seen in any of the cultures. Neither the mitochondria nor any other granules were concerned in the formation of the port-wine-colored areas. It seemed as though the material which exhibited the typical port-wine color was diffuse throughout certain parts of the cytoplasm and became more definitely localized during the death of the cell, which occurred coincidentally with the iodine

staining. Thus it is seen that some substance, which reacts as does glycogen when exposed to iodine, is present in the cells of fundulus cultures. It is possible that this substance is glycogen. That the substance which became port-wine colored was not the dextrose itself is shown by the fact that dextrose placed in Locke's solution did not so stain when exposed to iodine, while glycogen did.

A few cultures of chick embryos were tested in the same manner for a comparison with those of the fish embryos. No port-wine color was observed in the cells of the older embryos except in one somewhat degenerate culture where a few blebs were already present on certain of the dying cells; these blebs became slightly pink but in no case was there the port-wine coloration such as occurs in fundulus cultures. On the other hand the cells in cultures of very young embryos (48 hours) sometimes contained an abundance of this substance.

The Influence of Starch Upon the Amount of Glycogen Present in the Cells.

Soluble starch (Kaulbaum) was added to the medium of the cultures of fundulus embryos in order to determine whether it could be utilized by the cells to store up glycogen. The starch was dissolved in distilled water and boiled for two minutes; 60 c.c. of the starch solution was then added to 40 c.c. of sea water and the medium prepared in the same manner as for normal cultures. When the quantity of starch was less than 0.1 per cent. it had no appreciable effect upon the cells. In these cultures the growth was normal and no increase in the amount of glycogen could be detected. When larger amounts of starch were added, or when the starch became slightly clumped into masses of very small granules, as sometimes happened, small particles were occasionally taken up by the cell and appeared within the cytoplasm as small granules or granular masses, in some cases surrounded by a vacuole. Upon exposure to iodine the starch became blue, whether within the cell or in the medium. The surrounding vacuole became pale blue, or sometimes lilac, but never the port-wine color indicative of glycogen. The port-

wine color was present in the same regions as in normal cultures, but never greater in amount and sometimes less than in the normal control preparations. Even after a number of days the starch did not become changed into glycogen as, for instance, one 8-day-old culture in 0.75 per cent. starch solution, exposed to iodine, exhibited cells in which there were a few vacuoles some of which contained blue granules. The vacuoles were pale blue or lilac but never port-wine color.

Influence of Dextrose in the Medium Upon the Amount of Glycogen in the Cells.

Cultures were prepared in a medium free from dextrose in order to ascertain whether the lack of dextrose would prevent the appearance of glycogen in the cytoplasm of the cells. The results from twenty such cultures show that, while the amount of glycogen could be decreased by the lack of dextrose in the medium, its presence could not be entirely inhibited. Some of these cultures (48 to 72 hours) exhibited only a slight trace, if any, of the port-wine color when exposed to iodine. On the other hand, a few did contain decided evidences of a small quantity of this substance.

Explants into media containing 0.5 per cent., 1 per cent. and 2 per cent. dextrose, made at the same time as those without dextrose, showed a decided increase in the quantity of glycogen up to a certain point. In no instance did all of the cells of a culture exhibit the port-wine color. Neither did any one cell become greatly filled with this substance. Of all the cultures, those grown in a solution containing 2 per cent. dextrose exhibited the most marked amount of the port-wine-colored material; that is, more cells contained this substance, practically all of the fan-shaped processes had regions which were stained port-wine color, and the color was deeper in tone and did not fade as rapidly as in the normal cultures. Saliva placed upon cultures in 2 per cent. dextrose prevented the appearance of the port-wine color, just as it did in the normal cultures. In these experiments with different amounts of dextrose it was impossible to predict whether a given cell would show the port-wine color in

the presence of iodine. The cytoplasm was not characterized by any structure indicative of this substance, but appeared the same in all of the cells. The processes of the mesenchyme cells had the peculiar lighter areas which in some cases became port-wine colored and in others remained pale yellow.

Summary.

The cells of *Fundulus heteroclitus* grown in tissue cultures contain some substance which behaves in the same manner as does glycogen in the presence of iodine. It is possible that this substance may be glycogen.

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